Towards implementation of the tumour-stroma ratio in colorectal cancer

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Chapter 3

The tumour-stroma ratio as predictive aid towards a biopsy-based treatment strategy in rectal carcinoma

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HISTOPATHOLOGY. 2025 JUL;87(1):44-57

Abstract

Aims: Tumour-stroma ratio (TSR) scores of biopsy material in rectal carcinoma (RC) could aid a biomarker-based, upfront and personalized treatment strategy selection for RC patients. In a large retrospective, multicentre cohort, we aimed to validate the predictive value of biopsy-scored TSR on neoadjuvant therapy response, and secondary, disease-free and overall survival (DFS, OS).

Methods and results: Scanned haematoxylin-and-eosin-stained RC biopsy slides were collected from LUMC (N=116) and from the clinical PROCTOR-SCRIPT (N=142) and RAPIDO (N=271) trials. TSR was scored per protocol and categorized as stroma-low (≤50%) or stroma-high (>50%). Major response was defined as tumour regression grade (TRG) 1+2 by Mandard, including pathological complete response. Ultimately, a large and varied cohort with 373 RC patients was established. Locally advanced RC were more often stroma-high (P<0.001). We subsequently observed significantly less major response rates in the stroma-high RC after a neoadjuvant treatment approach (hazard ratio 0.63, 95% confidence interval 0.41 – 0.99; P=0.044). Despite correction for well-known risk factors in Cox hazard regression analysis, such as (y)pTNM-substages or residual tumour status, the TSR had no singular significant influence on DFS nor OS in multivariate analysis (P=0.438; P=0.934, respectively).

Conclusions: Biopsy-scored TSR can predict neoadjuvant therapy efficacy, as RC patients with stroma-high biopsies achieve less major response. Patient survival, however, is multifactorial, although response is an important predictor, influenced by TSR. Scoring TSR on RC biopsy material is a reliable histological parameter, implementation of which in treatment guidelines could aid to improve upfront selection for a watch-and-wait strategy.

Introduction

Optimalisation of therapeutic strategies in rectal carcinoma (RC) has been subject to many clinical trials over the years [1]. Management of RC has evolved, shifting the paradigm from initially aiming for enhanced locoregional control to whole organ preservation, rapidly improving patient-related outcomes like disease-free and overall survival (DFS and OS, respectively) [1, 2]. Currently, the cornerstone of international treatment guidelines encompasses risk stratification, based on disease extent as defined by the tumour-node-metastasis (TNM) classification [3], and clinical imaging factors like mesorectal fascia (MRF) involvement and/or extramural vascular invasion (EMVI) [4, 5]. Implementation of total mesorectal excision (TME) surgery [6] and preoperative treatment regimens [7-11] have led to optimal local control.

Starting with short course radiotherapy (SCRT) for improvement of locoregional control and survival [7], now, regimens including preoperative radiotherapy and chemotherapy, i.e. neoadjuvant therapy [10-12], has given rise to the watch-and-wait strategy [13], delaying and even potentially sparing patients burdensome surgery. However, response rates are prone to variation [14]. Moreover, heterogeneity is observed in reached clinical and pathological complete response (cCR and pCR, respectively) [10, 15-17]. With increasing RC incidence [4, 18], and high rates of treatment complications [4, 19], it is thus pivotal to improve upfront treatment selection. Current pathological risk factor parameters however focus mainly on the tumour epithelial compartment, i.e. neoplastic cells [4].

Convincing evidence is emerging that elements of the tumour microenvironment, especially the tumour stroma, are of detrimental influence of tumour behaviour, promoting tumour invasion and metastasis [20, 21]. Capturing this effect, the tumour-stroma ratio (TSR) is a robust and cost-effective histopathological parameter based on intratumoural stromal percentages [22]. The TSR has been validated as an independent biomarker in multiple tumour types: indeed, stroma-high (>50% stroma) gastrointestinal tract carcinomas not only have worse OS and DFS [23-25], as observed in colon carcinoma in the recently published prospective international UNITED study [26, 27], but also predicts worse response to (neo)adjuvant therapy [28-31]. Albeit often collectively termed, RC is a different entity than colon carcinoma [4]. Literature on TSR in RC specifically is scarce and mostly consists of relatively older, single centred RC series with limited patients and/or treatment types [25, 32-34].

To address this knowledge gap, we integrated two prominent clinical trials, i.e. PROCTOR-SCRIPT [9] and RAPIDO [10], with our local cohort. This collaboration enabled us to create an extensive overview

of the TSR in varied RC patient populations. A more biomarker-based approach on biopsies is crucial to improve future selection of responders and the TSR could aid in this prediction as literature has shown [32, 33]. As primary endpoint, we assessed the correlation between TSR and neoadjuvant therapy response. This study analysed the predictive potential of biopsy-scored TSR on DFS and OS as secondary endpoints. We hypothesized that the more aggressive and resistant stroma-high tumours would reach a response less often, and would have worse DFS and OS compared to their stroma-low counterparts, potentially influencing patient selection for a watch-and-wait strategy in the future.

Methods

Patient cohorts

Our local cohort (Leiden University Medical Centre, LUMC; N=116) was combined with available material from two well-established, independent clinical validation cohorts, i.e. subgroups of the PROCTOR-SCRIPT [9] (N=142) and RAPIDO [10] (N=271) trials. Hence, a large series comprising various TNM-stages and treatment regimens was analysed. All cohorts included patients ≥18 years with given informed consent. Additional inclusion and exclusion criteria for the clinical trials are mentioned in previous reports [9, 10]. Summarizing, the PROCTOR-SCRIPT was a combined study assessing the role of adjuvant chemotherapy compared to observation in RC patients treated with neoadjuvant therapy consisting of (chemo)radiotherapy and TME, whereas the RAPIDO analysed different neoadjuvant regimes in locally advanced RC (LARC). The LUMC cohort consisted of consecutive patients with a variety of (neo)adjuvant therapy types, of which available material was collected from patients operated after year 2000 with stage I-III RC and no previous malignancy <10 years prior to current RC.

Of note, PROCTOR-SCRIPT patients were included postoperatively, hence clinical TNM-stage nor clinical risk factors were not registered in the study database, although one inclusion criterium was pathological TNM-stage II/III. Stage II/III was given in case no imaging is performed and locoregional extent of disease was uncertain, e.g. in earliest included patients. Moreover, as different versions of the TNM classification were used, for optimal grouping and comparison, all variables are converted to TNM version 5 (1997). Age was registered at randomisation (PROCTOR-SCRIPT and RAPIDO) or diagnosis (LUMC). Supplementary Table 1 gives a detailed overview of treatment types and regimens per cohort.

Materials and tumour-stroma analysis

Scanned haematoxylin-and-eosin (H&E)-stained slides of diagnostic biopsies were collected at LUMC (LUMC, PROCTOR-SCRIPT) or requested from Radboud University Medical Centre (Radboudumc; RAPIDO). At LUMC, slides were scanned with the Panoramic-250 scanner, Radboudumc used the Panoramic-1000 (3DHistech, Hungary; 20x magnification). Analysis was performed with 3DHistech CaseViewer software (v2.7). Two independent observers (MP-GvP: LUMC, PROCTOR-SCRIPT; MP-DH: RAPIDO) scored the TSR on biopsies according to van Pelt *et al*. [35], blinded for clinical data. Subsequently, categorisation in stroma-low (≤50% stroma) and stroma-high (>50% stroma) followed (Figure 1, created in BioRender.com). Neoadjuvant therapy response was assessed on resection material through the tumour regression grade (TRG) in five categories as defined by Mandard [36] by local pathologists (LUMC, PROCTOR-SCRIPT) or in three groups (no-partial-complete response; RAPIDO). To ascertain the predictive correlation to TSR, TRG was dichotomized in clinically relevant and previously also maintained groups of TRG1+2 (including pCR and major responders) and TRG3-5 (non-major responders) [37].

Ethical considerations

Use of anonymised material from the studies PROCTOR-SCRIPT and RAPIDO were approved by the steering committees. These trials were conducted according to the Declaration of Helsinki (2013). All already available archival material and data of the LUMC cohort were coded and handled according to the Dutch National Ethical Guidelines ("Code of proper secondary use of human tissue"). No informed consent was necessary under the legislation for this retrospective analysis.

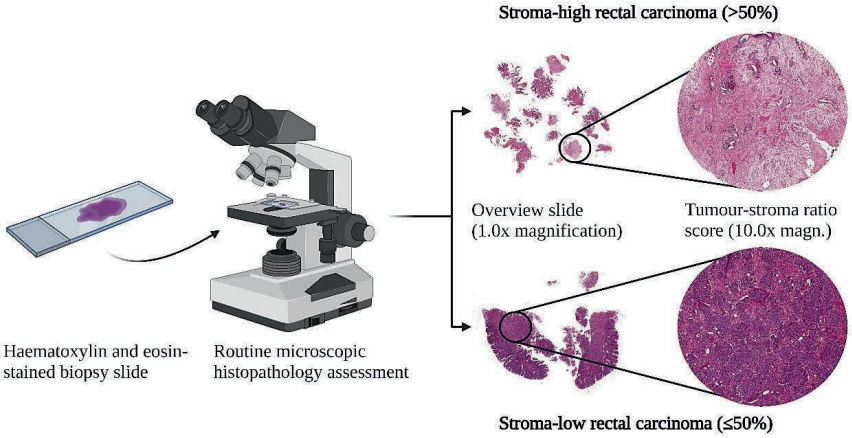

Figure 1. The process of scoring the tumour-stroma ratio (TSR) on haematoxylin and eosin-stained biopsy material using light microscopy.

First, using 1.0 – 2.5x magnification for a general overview of the complete slide, the area with the highest amount of tumour stroma is selected. Subsequently, the TSR is scored on a 10.0x magnifying objective, as per protocol of van Pelt *et al.* Finally, the biopsy is categorised as stroma-high (>50% intratumoural stroma; example shown above) or stroma-low (≤50% intratumoural stroma; example shown below). Created in BioRender.com.

Statistical analysis

Interobserver agreement Cohens kappa's were calculated between TSR biopsy scores. Assessment of prediction of TSR to neoadjuvant therapy response was done per TSR category and per therapy type subsequently. DFS was defined as period between date of surgery until any first event, i.e. recurrence (locoregional recurrence or distant metastasis), death (any cause) or until censoring. OS pertained to the period between date of surgery until death (any cause) or censoring. Censoring occurred when patients were disease-free and/or alive at last registration or after 10 years of follow-up.

Chi-square tests for nominal, Goodman Kruskal gamma statistics for ordinal, and Student's Independent T-tests for continuous variables were performed. Median follow-up time was calculated with the reversed Kaplan-Meier method, survival analyses were performed with Kaplan-Meier analyses and associated log-rank tests. Cox regression analysis for hazard ratio's (HR) with 95% confidence intervals

(CI) were calculated in univariate analysis for major response (event defined as pCR/TRG1+2), for the period between surgery and diagnosis (LUMC), first radiotherapy dose (PROCTOR-SCRIPT), or randomisation (RAPIDO). The variables significant of influence (P<0.05) in univariate analysis, were included in the multivariate analysis.

Continuous variables were expressed in means with standard deviations (SD), whereas nominal and ordinal variables were stated as number of frequencies and corresponding percentages. Two-tailed P-values <0.05 were considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics (v29.0).

Results

Patient cohorts

Establishing the final patient population, exclusions followed from the three cohorts, e.g. per(i)operative pathological stage IV (N=18) or absence of pathological data (N=68). In total, 373 RC patients were ultimately included in this study (Figure 2). Baseline characteristics of the combined cohort and those separately are presented in Table 1. There are differences between cohorts, inherently correlating to the used studies, including treatment type (P<0.001), clinical risk factors (P<0.001) and clinical TNM-stage (P<0.001). Most importantly, as could be expected due to the more aggressive nature of the tumours involved, the TSR is also already higher in the LARC patients of the RAPIDO, where 57% of patients were stroma-high, compared to approximately one-third in other cohorts and literature (P<0.001). Hereby, the full spectrum of presentations of RC is covered in our study.

Overview TSR analyses

The TSR scores had high Cohen's interobserver agreement kappa's of 0.84 (MP – DH; RAPIDO biopsies) and 0.77 (MP – GP; LUMC and PROCTOR-SCRIPT biopsies). Supplementary Table 2 presents an overview of stroma-low compared to stroma-high clinical variables in the total patient population and per cohort separately. Overall, although more clinical risk factors were found in stroma-high patients as expected (P=0.014), stroma-low patients had more often not undergone neoadjuvant therapy (P<0.001) and were operated on in earlier years (2009 vs. 2012 in stroma-high patients; P<0.001).

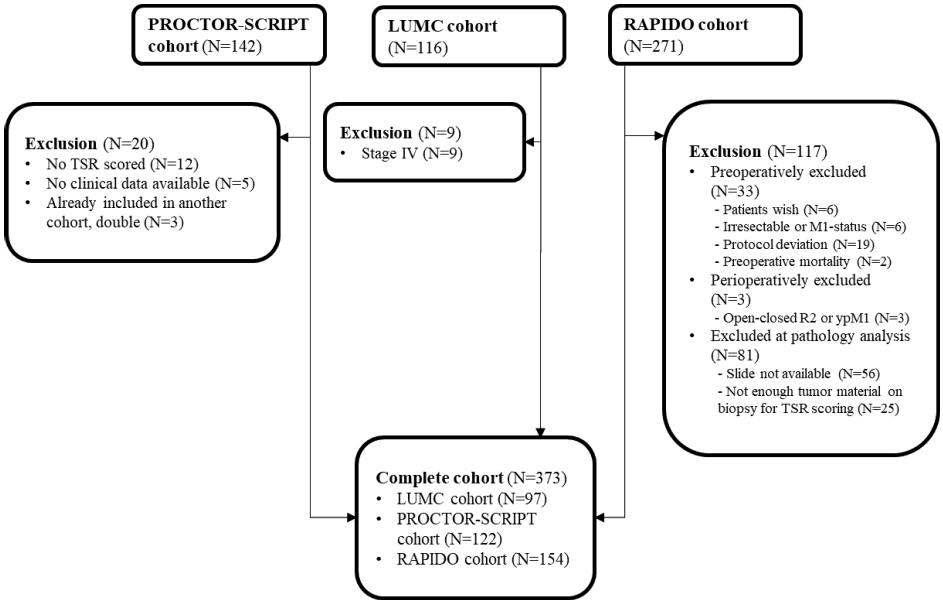

Figure 2. Flowchart showing the patient population with initial inclusion rates per separate cohort (PROCTOR-SCRIPT, LUMC or RAPIDO) and exclusion numbers and reasons, leading to the ultimately included final patient cohort (N=373). TSR, tumour-stroma ratio.

Table 1. Baseline characteristics of the eligible patients in the complete cohort and separate cohorts.

Baseline characteristics	**Complete cohort (N=373)**	**LUMC (N=97)**	**PROCTOR-SCRIPT (N=122)**	**RAPIDO (N=154)**	**P-value**
Participating centres					N/A
Total no. participating centres	28	1	22	13	
Sex					0.520#
Female	117 (31)	26 (27)	41 (34)	50 (33)	
Male	256 (69)	71 (73)	81 (66)	104 (68)	
Age					
Median age (years)	62 (55-69)	65 (57-73)	60 (55-68)	64 (54-69)	**0.017$**
Age of >70 years	75 (20)	29 (30)	19 (16)	27 (18)	**0.018#**
Treatment type					**<0.001#**
Neoadjuvant treatment and surgery	289 (78)	74 (76)	64 (52)	152 (99)	
Neoadjuvant treatment, surgery and adjuvant treatment	64 (17)	3 (3)	58 (48)	2 (1)	
Surgery and adjuvant treatment	4 (1)	4 (4)	0 (0)	0 (0)	
Surgery alone	16 (4)	16 (17)	0 (0)	0 (0)	
Clinical TNM-stage					**<0.001#**
II/III	4 (1)	4 (4)	N/A	0 (0)	
II	42 (11)	28 (29)	N/A	14 (9)	
III	202 (54)	62 (64)	N/A	140 (91)	
Unknown	*125 (34)*	*3 (3)*	*122 (100)*	*0 (0)*	
Clinical Locally Advanced Rectal Carcinoma (LARC)					**<0.001#**
No, no clinical LARC	23 (6)	23 (24)	N/A	0 (0)	
Yes, clinical LARC	221 (59)	67 (69)	N/A	154 (0)	
Unknown	*129 (35)*	*7 (7)*	*122 (100)*	*0 (0)*	

(*continued*) **Baseline characteristics**	**Complete cohort (N=373)**	**LUMC (N=97)**	**PROCTOR-SCRIPT (N=122)**	**RAPIDO (N=154)**	**P-value**
Tumour location					0.463#
Low rectum (<5cm anal verge)	133 (36)	42 (43)	40 (34)	51 (33)	
Mid rectum (5-10 cm anal verge)	108 (29)	27 (28)	34 (28)	46 (31)	
High rectum (>10 cm anal verge)	126 (34)	27 (28)	44 (36)	55 (36)	
Unknown	*6 (2)*	*1 (1)*	*4 (3)*	*2 (1)*	
Clinical risk factors					**<0.001#**
No, no additional risk factors	24 (10)	24 (26)	N/A	0 (0)	
Yes, 1 clinical risk factor present	48 (20	32 (35)	N/A	16 (10)	
Yes, 2 clinical risk factors present	85 (35)	19 (21)	N/A	66 (43)	
Yes, 3 or more clinical risk factors present	88 (36)	16 (18)	N/A	72 (47)	
Not (enough) determined, unknown	*128 (34)*	*6 (6)*	*122 (100)*	*0 (0)*	
Clinical risk factors					
Extramural venous invasion	19 (5)	1 (1)	N/A	18 (12)	
Mesorectal fascia involvement	95 (26)	16 (17)	N/A	79 (51)	
Clinical lateral lymph nodes	42 (11)	17 (18)	N/A	25 (16)	
Clinical T4-stage	38 (10)	7 (7)	N/A	31 (20)	
Clinical N+-stage	202 (54)	62 (64)	N/A	140 (91)	
Clinical N2-stage	*122 (33)*	*22 (23)*	*N/A*	*100 (65)*	
Tumour-stroma ratio biopsy					**<0.001#**
Stroma-low (≤50%)	215 (58)	69 (71)	80 (66)	66 (43)	
Stroma-high (>50%)	158 (42)	28 (29)	42 (34)	88 (57)	

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. Sum of percentages can be less or more than 100 due to rounding.

N/A, not applicable; TNM, tumour-node-metastasis

Calculated with Chi-square test. $ Calculated with one-way ANOVA analysis

TSR predictor of major response to neoadjuvant therapy

Pathological outcomes per TSR category of the total patient population and per therapy type separately are summarized in Supplementary Table 3. The CRT and RAPIDO regimens are combined in a large neoadjuvant treatment (NAT) group (N=182). Of note, SCRT was not intended to be used to reach pCR and only after more than 7 weeks after neoadjuvant therapy any significant downsizing can be seen[7, 38]. Hence, it almost mimics the situation in treatment-naïve patients: in stroma-high patients, more often a higher ypT-stage 2-4 (P=0.043) and less response was seen (P=0.044). Analysing major response rates per treatment type in-depth subsequently, the biopsy-scored TSR emerged as a valuable predictor, as stroma-high patients reached significantly less major response to NAT than stroma-low patients (HR 0.63, 95%CI 0.41 – 0.99; P=0.044) (Table 2).

Table 2. Univariate Cox regression analysis on the hazard of major response of TSR in total cohort and per treatment type.

Therapy type	**Variable**	**Number major responders (%)**	**Major response - Univariate analysis***		
			Hazard ratio	**95% Confidence interval**	**P-value**
Complete cohort (N=337)**	Stroma-low (N=188)	39 (21)	1		0.071
	Stroma-high (N=149)	41 (28)	0.666	0.428 – 1.035	
NAT (CRT + RAPIDO; N=182)	Stroma-low (N=81)	38 (47)	1		**0.044**
	Stroma-high (N=101)	40 (40)	0.632	0.405 – 0.989	
CRT (N=107)	Stroma-low (N=56)	26 (46)	1		0.308
	Stroma-high (N=51)	17 (33)	0.726	0.393 – 1.343	
RAPIDO (N=75)	Stroma-low (N=25)	12 (48)	1		0.839
	Stroma-high (N=50)	23 (42)	0.930	0.461 – 1.877	

CRT, chemoradiation (25x1.8-2 Gray and capecitabine monotherapy); N/A, not applicable; NAT, neoadjuvant treatment; RAPIDO (5x5 Gray followed by 6 cycles capecitabine and oxaliplatin).

*The period between surgery (for pathology) and diagnosis (LUMC), first radiotherapy dose (PROCTOR-SCRIPT), or randomisation (RAPIDO). The event is defined as major response (TRG1+2/pathological complete response).

**Complete cohort here pertains to those patients who had undergone neoadjuvant therapy and with a known and determined response

Survival analyses

To assess the predictive value of the TSR on DFS and OS, Kaplan-Meier analysis with log rank tests were first performed for the complete cohort and separate therapy groups (Supplementary Table 4; Supplementary Figure 1-2). No significant influence of the TSR was observed here. Subsequently, we used Cox hazard regression in the complete cohort. In univariate analysis, higher (y)pT and/or (y)pN stages, as well as residual tumour status or not reaching a major response were so significantly of influence, it potentially introduced bias and the TSR reached no significance (DFS P=0.800; OS P=0.856) (Table 3). Aiming to analyse the effect of the TSR relatively to the other, well-known, risk factors, we added the TSR as variable in the multivariate analysis. However, even correcting for these variables, the TSR did not assert an additional influence here either (DFS P=0.438; OS P=0.934).

Table 3. Univariate and multivariate analysis of disease-free survival and overall survival with Cox regression analysis in the complete cohort.

Variable (unit)	Disease-free survival						Overall survival					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	Hazard ratio	95% Confidence interval	P-value	Hazard ratio	95% Confidence interval	P-value	Hazard ratio	95% Confidence interval	P-value	Hazard ratio	95% Confidence interval	P-value
Sex				N/A						N/A		
Male	1		0.781				1		0.611			
Female	0.951	0.667-1.356					0.897	0.590-1.363				
Age at surgery - older category												
≤70 years of age	1		**0.022**	1		0.136	1		**0.001**	1		**<0.001**
>70 years of age	1.534	1.063-2.238		1.624	0.858-3.074		2.004	1.323-3.306		2.310	1.431-3.731	
Tumour location				N/A								
High rectum (>10 cm anal verge)	1		0.170				1		0.051	1		0.425
Mid rectum (5-10 cm anal verge)	1.257	0.831-1.902	0.278				1.651	1.000-2.726	**0.050**	1.137	0.589-2.193	0.702
Low rectum (<5cm anal verge)	1.500	0.983-2.289	0.060				1.849	1.109-3.083	**0.018**	1.430	0.803-2.545	0.224
Clinical risk factors				N/A						N/A		
No, no additional risk factors	1		0.763				1		0.385			
Yes, 1 or more clinical risk factors present	1.119	0.540-2.319					1.451	0.627-3.359				
Neoadjuvant therapy received				N/A						N/A		
Yes, received	1		0.731				1		0.444			
No, not received	0.875	0.409-1.871					0.703	0.286-1.730				
Received therapy, grouped				N/A						N/A		
NAT	1		0.373				1		0.520			
SCRT	0.995	0.455-2.173	0.990				0.778	0.308-1.962	0.595			
No neoadjuvant therapy	1.269	0.901-1.786	0.173				1.194	0.799-1.782	0.387			
Adjuvant treatment				N/A						N/A		
Yes, adjuvant treatment received	1		0.384				1		0.704			
No, no adjuvant treatment received	0.830	0.546-1.263					0.904	0.538-1.521				
Surgery type, grouped				N/A								
Low anterior resection (LAR)	1		0.511				1		**0.012**	1		0.114
Abdominoperineal resection (APR)	1.219	0.870-1.709	0.250				1.753	1.181-2.602	**0.005**	1.641	0.966-2.789	0.067
Hartmann	1.157	0.503-2.660	0.731				2.090	0.892-4.896	0.090	1.924	0.651-5.690	0.237

(continued)											
Residual tumour (Wittekind 2009)											
R0 resection	1		**<0.001**	1		0.493	1		**<0.001**	1		**<0.001**
R+ resection	3.082	1.874-5.069		1.354	0.569-3.220		3.367	1.907-5.946		3.401	1.782-6.490	
Major response												
TRG1+2	1		**<0.001**	1		0.275	1		**0.017**	1		0.701
TRG3-5	2.449	1.466-4.092		3.407	0.377-30.83		1.968	1.129-3.432		1.183	0.501-2.793	
y(p)T-category												
y(p)T-stage 0+1	1		**<0.001**	1		0.973	1		**0.023**	1		0.398
y(p)T-stage 2-4	2.748	1.520-4.966		N/A	N/A		2.065	1.105-3.860		1.521	0.575-4.020	
Lymph nodes (LN)				N/A						N/A		
LN examined ≥12	1		0.716				1		0.995			
LN examined <12	0.941	0.676-1.308					1.001	0.682-1.471				
y(p)N-category												
y(p)N-stage 0	1		**<0.001**	1		0.210	1		**<0.001**	1		**0.004**
y(p)N-stage +	2.694	1.900-3.819		1.678	0.748-3.764		2.152	1.452-3.187		2.117	1.274-3.517	
Differentiation grade tumour				N/A						N/A		
Low grade (well-moderate)	1		0.691				1		0.152			
High grade (poor-undifferentiated)	1.124	0.631-2.002					1.564	0.849-2.882				
Pathology risk factors										N/A		
No risk factors present	1		**0.005**	1		0.056	1		0.118			
Yes, 1 or more risk factors present	2.301	1.290-4.107		1.889	0.983-3.623		1.746	0.868-3.513				
TSR biopsy												
Stroma-low	1		0.800	1		0.438	1		0.856	1		0.934
Stroma-high	1.044	0.750-1.453		0.798	0.451-1.412		1.036	0.705-1.523		0.982	0.637-1.512	

N/A, not applicable. NAT, neoadjuvant treatment. SCRT, short course radiotherapy. TNM, tumour-node-metastasis classification. TRG, tumour regression grade. TSR, tumour-stroma ratio. Disease-free survival and overall survival are censored at 10 years. Variables with a significant (P≤0.050) influence on outcome in univariate analysis are included in multivariate analysis.

Discussion

The present study set out to determine the predictive effect of biopsy-scored TSR on response to neoadjuvant therapy. In a large and varied multicentre patient population, we first observed that LARC, characterised through imaging as a more aggressive and invading tumour, was significantly more often categorised as stroma-high. We subsequently validated the TSR's predictive value on response. In RC patients undergoing a NAT approach, significantly less major response rates were achieved in those with a stroma-high biopsy. Identifying potential major therapy responders can ultimately aid upfront selection of patients for treatment strategies, e.g. watch-and-wait.

As secondary endpoint, the prognostic effect on DFS and OS of the TSR in RC patients was assessed. Despite correction for the significant influence of well-known risk factors like (y)pTNM-substages and residual tumour status, the TSR did not significantly assert a singular predictive influence on DFS or OS in our cohort. Response however, remained an important prognosticator, to which the TSR is a major contributor. This is most likely due to the presence of large multifactorial causal relations of various risk factors on survival, such as increasing sequential treatment options, underlining the need for a multidisciplinary and patient-tailored approach [4]. Moreover, underlying biological processes influencing response and tumour behaviour such as mutational status, are merely determined in a minority of included patients but increasingly analysed in current practices [39].

The past decades, the traditional neoplastic cell-centred view has incrementally been expanded to include the surrounding tumour microenvironment [40, 41]. The complex interaction between these entities has hence been subject to increasing research [20, 42]. In 2007, our research group first explored the absolute ratio of the tumour epithelium compartment compared to the stromal compartment: the TSR [23]. Since then, the field of the TSR has exponentially gained interest, worldwide [22, 24, 25, 28, 30, 43]. Recently, the detrimental influence of tumour stromal abundance on patient-related outcomes was proven in our UNITED study, where stroma-high colon carcinomas indeed led to worse DFS [26, 27]. This parameter has therefore been proposed [27] to be implemented in international guidelines, for instance in the TNM-classification.

Although the TSR had already previously proven to be of predictive value in biopsies and RC [32, 33, 44], large, novel and multicentre studies were lacking [34, 45]. As upfront selection of RC patients for personalised therapeutic strategies is gaining importance with the shift towards a watch-and-wait strategy [2, 4, 46], implementing biopsy-based biomarkers to aid this selection is pivotal. Potential

implications of our results, proving that biopsy-scored stroma-high RC patients are less prone to reach major response in a NAT approach, could be far-reaching. Including the TSR in the panel with all other important clinicopathological variables currently used by multidisciplinary meeting, could lead to an improved tailored approach, where in a shared-decision making setting, patients could even be advised to be spared the potentially less efficient though burdensome treatment and instead be recommended for immediate surgery, especially in frail elderly with comorbidities.

The largest strength but simultaneously also limitation of this study was inherent to the choice for a large and varied patient population; the differences between the cohorts prohibited large pooling as this could lead to skewed results. Of interest, it was for example observed that the RAPIDO trail, including only LARC patients, had significantly more stroma-high patients than the other cohorts with less advanced tumours. While this also emphasizes the importance of the stromal compartment and influence of its abundance on tumour invasiveness, it also gave rise to potential selection bias. Therefore, we aimed to answer our research questions in specific, though smaller, subgroups. Another limitation pertains to the retrospective aspect of this study. Moreover, despite the use of large clinical trials, these are still somewhat dated. With the rise of various neoadjuvant approaches [47], scoring more recent trials or even aiming to include the TSR scored on biopsies as parameter in prospective or randomized controlled trials in the future would therefore be imperative.

The current ESMO guidelines in management of RC state that there are several regression grade classifications including the Mandard [36] and the Dworak [48] classifications without indicating an optimal method for scoring tumour regression. Despite the fact that the Mandard classification may be not the most widely used for determining tumour regression and TRG has been described to potentially underestimate tumour shrinkage compared to e.g. a fragmentation pattern [49], many studies, including ours, use the Mandard score [32], which enhances potential comparison. In any case, a reliable score should include a ‘complete-partial-no response’ category [4]. Although the International Collaboration on Cancer Reporting (ICCR) colorectal pathology guidelines [50] mention another, the Ryan four-tier system [51], ultimately, all classifications for scoring result in similar endpoints.

Future studies including more parameters of the tumour microenvironment as well could enhance our understanding on the dynamic interplay between the various components of this entity. The immune cell component [52-54], tumour budding [55], or biomarkers such as circulating tumour DNA (ctDNA) and other liquid biopsy methods show promise in colon [56, 57] as well as RC [58], and could provide valuable insights. TSR is, in contrast to most biomarkers, uncomplicated and cost-effective [35], taught

by E-learning [59] and hence standardized with high interobserver agreement [60]. Furthermore, in this study, we collected scanned H&E stained biopsy slides. Artificial intelligence is exponentially being researched in pathology, nowadays successfully completing high-performance tasks like classification [61]. Future studies could use this collection for developing algorithms, e.g. supervised deep learning models trained by pathologists' annotations to characterise tissue types [62], resulting in automatised TSR quantification in biopsies, supporting pathologists' workload. Currently, some studies show promise in various TSR scoring methods, mostly performed on primary tumours in colon [63-65]. However, for RC, studies are still scarce [66], even less aiming to predict response from TSR in biopsies [67]. Moreover, unsupervised learning could potentially discover novel histological patterns on unannotated slides, improving personalized predictions on therapy response and survival [68].

We have hereby presented a large and multicentre study validating the predictive significance of biopsy-scored TSR on neoadjuvant therapy response in RC. Using well-established clinical trials, we conclude that patients with stroma-high RC biopsies will reach less major response in a NAT approach. The TSR should thus be implemented in routine pathology diagnostics and the current clinicopathological panel of parameters, which multidisciplinary meetings consider for RC patients in personalized selection of treatment strategies.

Acknowledgements

We thank the PROCTOR-SCRIPT and RAPIDO steering committees for use of their data and support. We also want to thank pathologist Arantza Farina Sarasqueta for aiding in the initial scoring procedures. We thank Sonay Kuş Öztürk (Radboudumc) for scanning of the RAPIDO slides, and Ronald van Vlierberghe and Geeske Dekker-Ensink (both LUMC) for scanning of the PROCTOR-SCRIPT and LUMC slides, as well as laboratory support.

Funding

This work was supported by the Bollenstreekfonds, Hillegom, the Netherlands (no grant number). This funder had no role in study design, data collection and analysis, nor in the decision to publish, nor in the preparation of the manuscript. The original PROCTOR-SCRIPT trial was supported by the Dutch Cancer Society (KWF 1999-03 and KWF 2003-16), the Dutch Colorectal Cancer Group, and the Swedish Cancer Society. Furthermore, Roche has provided an unrestricted educational grant. The original RAPIDO trial was funded by the Dutch Cancer Foundation, Swedish Cancer Society, Spanish Ministry of Economy and Competitiveness, and Spanish Clinical Research Network.

Clinical trial registration

PROCTOR-SCRIPT: Dutch Colorectal Cancer group, CKTO 2003-16, ISRCTN36266738

RAPIDO: EudraCT 2010-023957-12, and ClinicalTrials.gov NCT01558921

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Supplementary Table 1. Detailed overview of the treatment types within the three separate cohorts.

Cohort	Treatment group	Neoadjuvant therapy	Adjuvant therapy
LUMC (N=97)	Neoadjuvant + surgery (N=74)	SCRT (N=52); CRT (N=17); Other (N=5)	N/A
	Neoadjuvant + surgery + adjuvant (N=3)	SCRT (N=3)	PROCTOR (N=2); SCRIPT (N=1)
	Surgery + adjuvant (N=4)	None	PROCTOR (N=1); Radiotherapy (N=2); CAPOX (N=1)
	Surgery alone (N=16)	None	N/A
PROCTOR-SCRIPT (N=122)	Neoadjuvant + surgery (N=64)	SCRT (N=59); CRT (N=5)	N/A
	Neoadjuvant + surgery + adjuvant (N=58)	SCRT (N=52); CRT (N=6)	
	Surgery + adjuvant (N=0)	N/A	
	Surgery alone (N=0)	N/A	N/A
RAPIDO (N=154)	Neoadjuvant + surgery (N=152)	RAPIDO (N=75); CRT (N=77)	N/A
	Neoadjuvant + surgery + adjuvant (N=2)	CRT (N=2)	Unknown (N=2)
	Surgery + adjuvant (N=0)	N/A	N/A
	Surgery alone (N=0)	N/A	N/A

CAPOX, capecitabine and oxaliplatin; CRT, chemoradiation (25x1.8-2 Gray and capecitabine monotherapy); N/A, not applicable; PROCTOR (5-FU + Leucovorin); RAPIDO (5x5 Gray followed by 6 cycles of CAPOX); SCRIPT (capecitabine monotherapy); SCRT, short course radiotherapy (5x5 Gray).

Supplementary Table 2. Characteristics of the biopsy-scored stroma-low compared to stroma-high patients in total cohort and per cohort.

Variables (unit)	**Total cohort (N=373)**			**LUMC (N=97)**			**PROCTOR-SCRIPT (N=122)**			**RAPIDO (N=154)**		
	Stroma-low (N=215)	**Stroma-high (N=158)**	**P-value**	**Stroma-low (N=69)**	**Stroma-high (N=28)**	**P-value**	**Stroma-low (N=80)**	**Stroma-high (N=42)**	**P-value**	**Stroma-low (N=66)**	**Stroma-high (N=88)**	**P-value**
Sex			0.209#			0.798#			0.653#			0.112#
Female	73 (34)	44 (28)		19 (28)	7 (25)		28 (35)	13 (31)		26 (39)	24 (27)	
Male	142 (66)	114 (72)		50 (73)	21 (75)		52 (65)	29 (69)		40 (61)	64 (73)	
Age*												
Mean age (in years; SD)	62 (10)	62 (9)	0.978$	64 (13)	66 (9)	0.372$	61 (9)	61 (9)	0.767$	62 (10)	61 (9)	0.605$
Age, category**			0.841#			0.856#			0.443#			0.854#
≤70 years of age	171 (80)	127 (80)		48 (70)	20 (71)		69 (86)	34 (81)		54 (82)	73 (83)	
>70 years of age	44 (21)	31 (20)		21 (30)	8 (29)		11 (14)	8 (19)		12 (18)	15 (17)	
Clinical TNM-stage***			0.103#			0.091#	N/A****					0.257#
II	27 (21)	15 (13)		23 (33)	5 (18)					4 (6)	10 (11)	
III	102 (79)	100 (87)		40 (58)	22 (79)					62 (94)	78 (89)	
*Unknown and II/III**** (excluded from analysis)*	*86 (40)*	*43 (27)*		*6 (9)*	*1 (4)*					*0 (0)*	*0 (0)*	
Tumour location			0.186#			0.611#			0.056#			0.827#
Low rectum (<5cm anal verge)	79 (37)	54 (35)		28 (41)	14 (50)		28 (35)	12 (29)		23 (66)	28 (32)	
Mid rectum (5-10 cm anal verge)	54 (26)	53 (34)		19 (28)	8 (29)		17 (21)	17 (40)		18 (27)	28 (32)	
High rectum (>10 cm anal verge)	78 (37)	48 (31)		21 (30)	6 (21)		33 (41)	11 (26)		24 (36)	31 (35)	
Unknown (excluded from analysis)	*4 (2)*	*3 (1)*		*1 (1)*	*0 (0)*		*2 (3)*	*2 (5)*		*1 (2)*	*1 (1)*	
Clinical risk factors*****			**0.014#**			0.229#	N/A*****					0.111#
No, no additional risk factors	20 (16)	4 (3)		20 (29)	4 (14)					N/A	N/A	
Yes, 1 clinical risk factor present	23 (18)	25 (22)		20 (29)	12 (43)					3 (5)	13 (15)	
Yes, 2 clinical risk factors present	40 (31)	45 (39)		11 (16)	8 (29)					29 (44)	37 (42)	
Yes, 3 or more clinical risk factors present	46 (36)	42 (36)		12 (17)	4 (14)					34 (52)	38 (43)	
Not (enough) determined, unknown (excluded from analysis)	*85 (40)*	*42 (27)*		*6 (9)*	*0 (0)*					*0 (0)*	*0 (0)*	
Treatment type			**<0.001#**			0.074#			0.130#			0.100#
Neoadjuvant treatment and surgery	151 (70)	139 (88)		49 (71)	25 (89)		38 (48)	26 (62)		64 (97)	88 (100)	
Neoadjuvant treatment, surgery and adjuvant treatment	47 (22)	16 (10)		3 (4)	0 (0)		42 (53)	16 (38)		2 (3)	0 (0)	
Surgery and adjuvant treatment	2 (1)	2 (1)		2 (3)	2 (7)		N/A	N/A		N/A	N/A	
Surgery alone	15 (7)	1 (1)		15 (22)	1 (4)		N/A	N/A		N/A	N/A	

(continued)												
Neoadjuvant treatment received												
No, no neoadjuvant treatment	17 (8)	3 (2)	**0.011#**	17 (25)	3 (11)	0.125#	N/A	N/A		N/A	N/A	
Yes, neoadjuvant treatment received, of which	198 (92)	155 (98)	**<0.001#**	52 (75)	25 (89)	0.350#	80 (100)	42 (100)	0.141#	66 (100)	88 (100)	**0.020#**
Short course radiotherapy (SCRT; 5x5 Gray)	114 (58)	52 (34)		39 (80)	16 (70)		75 (94)	36 (86)		N/A	N/A	
Chemoradiation therapy (CRT; 28x1.2 Gray and capecitabine monotherapy)	56 (28)	51 (33)		10 (20)	7 (30)		5 (6)	6 (14)		41 (62)	38 (43)	
RAPIDO-regime (5x5 Gray followed by 6 cycles capecitabine and oxaliplatin [CAPOX])	25 (13)	50 (32)		N/A	N/A		N/A	N/A		25 (38)	50 (57)	
Other (excluded from analysis)	*3 (2)*	*2 (1)*		*2 (2)*	*2 (2)*		*0 (0)*	*0 (0)*		*0 (0)*	*0 (0)*	
Waiting period before surgery												
Mean duration period (in weeks; SD)	10 (9)	15 (10)	**<0.001$**	10 (7)	11 (7)	0.883$	2 (3)	3 (4)	0.227$	20 (6)	22 (6)	**0.032$**
Period shorter <12 weeks	129 (60)	58 (37)	**<0.001#**	51 (74)	20 (71)	0.802#	78 (98)	38 (91)	0.088#	N/A	N/A	N/A
Period longer ≥12 weeks	86 (40)	100 (63)		18 (26)	8 (29)		2 (3)	4 (10)		66 (100)	88 (100)	
Surgery year												
Mean surgery year (SD)	2009 (5)	2012 (4)	**<0.001$**	2007 (5)	2010 (5)	**0.027$**	2006 (3)	2007 (3)	0.449$	2015 (1)	2015 (1)	0.500$
Surgery type, grouped			0.174#			0.710#			0.721#			0.089#
Low anterior resection (LAR)	122 (57)	84 (53)		35 (51)	13 (46)		49 (61)	22 (52)		38 (58)	49 (56)	
Abdominoperineal resection (APR)	85 (40)	60 (38)		32 (46)	15 (54)		28 (35)	17 (40)		25 (38)	28 (32)	
Hartmann	5 (2)	11 (7)		1 (1)	0 (0)		3 (4)	2 (5)		1 (2)	9 (10)	
Other (excluded from analysis)	*3 (1)*	*3 (2)*		*1 (1)*	*0 (0)*		*0 (0)*	*1 (2)*		*2 (3)*	*2 (2)*	
Adjuvant treatment received			**0.005#**			0.986#			0.130#			0.100#
No, no adjuvant treatment	166 (77)	140 (89)		64 (93)	26 (93)		38 (48)	26 (62)		64 (97)	88 (100)	
Yes, adjuvant treatment received, of which	49 (23)	18 (11)	0.125#	5 (7)	2 (7)	0.208#	42 (53)	16 (38)	0.161#	2 (3)	0 (0)	N/A
5-FU and Leucovorin (PROCTOR)	*19 (9)*	*3 (2)*		*3 (60)*	*0 (0)*		*16 (38)*	*3 (19)*		*0 (0)*	*0 (0)*	
Capecitabine monotherapy (SCRIPT)	*27 (13)*	*13 (8)*		*1 (20)*	*0 (0)*		*26 (62)*	*13 (81)*		*0 (0)*	*0 (0)*	
Radiotherapy	*1 (1)*	*1 (1)*		*1 (20)*	*1 (50)*		*0 (0)*	*0 (0)*		*0 (0)*	*0 (0)*	
Capecitabine and oxaliplatin (CAPOX)	*0 (0)*	*1 (1)*		*0 (0)*	*1 (50)*		*0 (0*	*0 (0)*		*0 (0)*	*0 (0)*	
Missing	*2 (1)*	*0 (0)*		*0 (0)*	*0 (0)*		*0 (0)*	*0 (0)*		*2 (100)*	*0 (0)*	

All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. Sum of percentages can be less or more than 100 due to rounding. N/A, not applicable. SD, standard deviation. TNM, tumour-node-metastasis.

*Age at randomisation (PROCTOR-SCRIPT and RAPIDO) or diagnosis (LUMC).

**Age cutoff of 70 years is used here, averaging most trials.

***Different versions of the TNM classification were used, for optimal grouping and comparison, all variables are converted to TNM version 5 (1997).

****Stage II/III is given in case no imaging is performed and locoregional extent of disease is uncertain.

PROCTOR-SCRIPT patients were included after surgery, hence clinical TNM-stage or risk factors were not registered in the study database, although inclusion criteria was pathological TNM-stage II/III

*****Clinical risk factors as determined by guidelines to assess locally advanced rectal carcinoma (e.g. ESMO guidelines), see below overview of all.

Clinical risk factors were not always determined on imaging, especially in patients operated before 2010.

*****Multiple risk factors may exist simultaneously, hence percentages do not add up to 100. Not always determined on imaging, especially in patients operated before 2010.

Calculated with Chi-square test

$ Calculated with the Student's Independent Samples T-test

Supplementary Table 3. Overview of the pathology outcomes between biopsy-scored stroma-low compared to stroma-high patients in the total cohort and per treatment type.

Variables (unit)	Total cohort (N=373)			No neoadjuvant therapy (N=20)			SCRT (N=166)			NAT (N=182)		
	Stroma-low (N=215)	**Stroma-high (N=158)**	**P-value**	**Stroma-low (N=17)**	**Stroma-high (N=3)**	**P-value**	**Stroma-low (N=114)**	**Stroma-high (N=52)**	**P-value**	**Stroma-low (N=81)**	**Stroma-high (N=101)**	**P-value**
Residual tumour*			0.441#			N/A			0.720#			0.649#
R0 resection	202 (94)	146 (92)		17 (100)	3 (100)		106 (94)	48 (92)		76 (94)	93 (92)	
R+ resection	12 (6)	12 (8)		0 (0)	0 (0)		7 (6)	4 (8)		5 (6)	8 (8)	
Missing, unknown (excluded from analysis)	*1 (0)*	*0 (0)*		*0 (0)*	*0 (0)*		*1 (1)*	*0 (0)*		0 (0)	0 (0)	
Minimal distance to circumferential margin (CRM)			0.280$			0.802$			0.936$			0.188$
Mean CRM distance (in millimetres; SD)	11 (12)	10 (9)		16 (13)	14 (11)		13 (14)	13 (12)		7 (4)	8 (5)	
(y)pTNM-stage**			0.058ŧ			0.815ŧ			**0.049ŧ**			0.441ŧ
(y)pTNM-stage 0	22 (10)	22 (14)		N/A	N/A		N/A	N/A		22 (27)	22 (22)	
(y)pTNM-stage I	23 (11)	21 (13)		7 (41)	1 (33)		10 (9)	3 (6)		10 (12)	23 (23)	
(y)pTNM-stage II	53 (25)	43 (27)		5 (29)	1 (33)		23 (20)	5 (10)		19 (24)	30 (30)	
(y)pTNM-stage III	117 (54)	72 (46)		5 (29)	1 (33)		81 (71)	44 (85)		30 (37)	26 (26)	
Maximum diameter tumour			0.280$			0.146$			0.093$			0.212$
Mean tumour diameter (in millimetres; SD)	30 (18)	38 (111)		39 (27)	25 (10)		36 (12)	40 (13)		18 (17)	37 (128)	
All y(p)T-categories**			0.384ŧ			0.678ŧ			0.175ŧ			0.998ŧ
y(p)T-stage 0	26 (12)	23 (15)		N/A	N/A		N/A	N/A		26 (32)	23 (23)	
y(p)T-stage I	8 (4)	5 (3)		1 (6)	0 (0)		4 (4)	0 (0)		3 (4)	5 (5)	
y(p)T-stage II	52 (24)	40 (25)		7 (41)	1 (33)		30 (26)	11 (21)		13 (16)	27 (27)	
y(p)T-stage III	114 (53)	83 (53)		8 (47)	2 (67)		77 (68)	39 (75)		29 (36)	41 (41)	
y(p)T-stage IV	15 (7)	7 (4)		1 (6)	0 (0)		3 (3)	2 (4)		10 (12)	5 (5)	
Combined y(p)T-categories**			0.627ŧ			0.348ŧ			**0.043ŧ**			0.245ŧ
y(p)T-stage 0+I	34 (16)	28 (18)		1 (6)	0 (0)		4 (4)	0 (0)		29 (36)	28 (28)	
y(p)T-stage II-IV	181 (84)	130 (82)		16 (94)	3 (100)		110 (97)	52 (100)		52 (64)	73 (72)	
Lymph nodes (LN)												
No. patients ≥ LN 12 examined	93 (44)	79 (50)	0.190#	11 (65)	2 (67)	0.948#	49 (43)	21 (41)	0.793#	33 (41)	55 (55)	0.066#
No. patients < LN 12 examined	121 (57)	78 (50)		6 (35)	1 (33)		64 (57)	30 (59)		48 (59)	46 (46)	
Missing (excluded from analysis)	*1 (0)*	*1 (0)*		*0 (0)*	*0 (0)*		*1 (1)*	*1 (2)*		*0 (0)*	*0 (0)*	

(continued)												
LN per patient												
Mean LN examined (SD)	12 (6)	12 (7)	0.269$	15 (6)	16 (9)	0.470$	11 (6)	11 (5)	0.438$	11 (5)	13 (8)	0.086$
Mean LN positive (SD)	2 (3)	1 (2)	**0.042$**	2 (4)	1 (2)	0.801$	2 (4)	3 (3)	0.659$	1 (2)	1 (1)	**0.014$**
y(p)N-category**			**0.030†**			0.876†			0.121†			0.051†
y(p)N-stage 0	98 (46)	86 (54)		12 (71)	2 (67)		33 (29)	8 (15)		51 (63)	75 (74)	
y(p)N-stage I	79 (37)	57 (36)		1 (6)	1 (33)		58 (51)	32 (62)		19 (24)	23 (23)	
y(p)N-stage II	38 (18)	15 (10)		4 (24)	0 (0)		23 (20)	12 (23)		11 (14)	3 (3)	
Differentiation grade tumour			0.135#			0.814#			0.629#			0.198#
Well to moderate (low grade)	140 (73)	110 (77)		11 (73)	2 (67)		88 (89)	43 (92)		41 (85)	64 (93)	
Poor to undifferentiated (high grade)	23 (12)	10 (7)		4 (27)	1 (33)		11 (11)	4 (9)		7 (15)	5 (7)	
Missing (excluded from analysis)	*25 (12)*	*15 (10)*		*2 (12)*	*0 (0)*		*15 (13)*	*5 (10)*		*47 (58)*	*71 (70)*	
N/A (no tumour; excluded from analysis)	*27 (13)*	*23 (15)*		*N/A*	*N/A*		*N/A*	*N/A*		*25 (31)*	*23 (23)*	
Pathology risk factors***												
Not determined	85 (40)	83 (53)		8 (47)	2 (67)		28 (25)	8 (15)		*47 (58)*	*71 (70)*	
Determined, and	105 (49)	52 (33)	0.757#	9 (53)	1 (33)	0.598#	86 (75)	44 (85)	0.932#	9 (11)	7 (7)	0.949#
No risk factors present	39 (37)	18 (35)		7 (78)	1 (100)		28 (33)	14 (32)		4 (44)	3 (43)	
Yes, 1 or more risk factors present	66 (63)	34 (65)		2 (22)	0 (0)		58 (67)	30 (68)		5 (56)	4 (57)	
N/A (no tumour; excluded from analysis)	*25 (12)*	*23 (15)*		*N/A*	*N/A*		*N/A*	*N/A*		*25 (31)*	*23 (23)*	
Tumour response****				N/A								
Determined, and	188 (87)	149 (94)	0.074†				105 (92)	47 (90)	**0.044†**			0.265†
Complete pathological response (pCR; TRG 1)	22 (12)	22 (15)					0 (0)	0 (0)		22 (27)	22 (22)	
Partial pathological response (pPR; TRG 2+3)	73 (39)	68 (46)					21 (20)	4 (9)		50 (62)	63 (63)	
No or nearly no pathological response (TRG 4+5)	93 (50)	59 (40)					84 (80)	43 (92)		9 (11)	16 (16)	
N/A (no neoadjuvant therapy; excluded from analysis)	*17 (8)*	*3 (2)*					*N/A*	*N/A*		*N/A*	*N/A*	
Not determined, unknown (excluded from analysis)	*10 (5)*	*6 (4)*					*9 (8)*	*5 (10)*		*N/A*	*N/A*	
Major response in those determined			0.150†	N/A					0.316†			0.322†
No, no major response (TRG3-5)	149 (79)	108 (73)					104 (99)	47 (100)		43 (53)	61 (60)	
Yes, major response (TRG1+2)	39 (21)	41 (28)					1 (1)	0 (0)		38 (47)	40 (40)	0.322†

Other type of NAT (N=5) than above is excluded at category for analysis purposes. All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. Sum of percentages can be less or more than 100 due to rounding.

N/A, not applicable; SD, standard deviation; SCRT, short course radiotherapy (5x5 Gray); TNM, tumour-node metastasis; NAT, neoadjuvant treatment, including chemoradiation (25x1.8-2 Gray and capecitabine monotherapy); and RAPIDO (5x5 Gray followed by 6 cycles capecitabine and oxaliplatin); TRG, tumour regression grade.

*Residual tumour according to Wittekind (2009).

**Different versions of the TNM classification were used, here all variables are converted to TNM version 5 (1997).

***Pathology risk factors include: extramural venous invasion, lymphatic invasion, (intramural) venous invasion, perineural invasion or combinations of these.

****Tumour response is categorized in three; no/partial/complete response, or based on TRG groups as categorized by Mandard.

Calculated using the Chi-square test. $ Calculated using the Student's Independent Samples T-test. ŧ Calculated using the Goodman and Kruskal's test for gamma.

Supplementary Table 4. Overview of the clinical outcomes between biopsy-scored stroma-low compared to stroma-high patients in the total cohort and per treatment type.

Variables (unit)	Total cohort (N=373)			No neoadjuvant therapy (N=20)			SCRT (N=166)			NAT (N=182)		
	Stroma-low (N=215)	Stroma-high (N=158)	P-value	Stroma-low (N=17)	Stroma-high (N=3)	P-value	Stroma-low (N=114)	Stroma-high (N=52)	P-value	Stroma-low (N=81)	Stroma-high (N=101)	P-value
Follow-up time - months			0.144ǂ			0.970ǂ			0.412ǂ			0.128ǂ
Mean follow-up time (in years; SE)	8.5 (0.4)	7.7 (0.3)		11.1 (1.8)	13.5 (4.9)		8.7 (0.6)	7.8 (0.7)		6.9 (0.3)	7.4 (0.2)	
Disease-free survival												
Mean disease-free survival time (in years; SE)	7.0 (0.3)	6.9 (0.3)	0.800§	7.7 (0.9)	3.3 (0.9)	0.093§	6.7 (0.4)	6.4 (0.6)	0.804§	6.8 (0.4)	7.3 (0.4)	0.980§
Events until censoring	81 (38)	62 (39)	0.759#	5 (29)	2 (67)	0.212#	48 (42)	21 (40)	0.835#	27 (33)	37 (37)	0.643#
Event type			0.690#			0.290#			0.424#			0.915#
Locoregional recurrence	11 (14)	11 (18)		0 (0)	0 (0)		4 (8)	2 (10)		6 (22)	9 (24)	
Distant metastasis	52 (64)	40 (65)		3 (60)	2 (100)		33 (69)	17 (81)		16 (59)	20 (54)	
Death, any cause	18 (22)	11 (18)		2 (40)	0 (0)		11 (23)	2 (10)		5 (19)	8 (22)	
Overall survival												
Mean overall survival time (in years; SE)	8.1 (0.2)	8.1 (0.2)	0.856§	8.8 (0.6)	8.1 (1.6)	0.773§	8.0 (0.3)	7.7 (0.5)	0.696§	8.1 (0.4)	8.4 (0.3)	0.764§
Deaths until censoring	60 (28)	46 (29)	0.798#	4 (24)	1 (33)	0.718#	34 (30)	16 (31)	0.902#	21 (26)	27 (27)	0.902#
Cause of death			0.979#			0.659#			0.658#			0.851#
(Metastases of) current cancer	38 (63)	30 (65)		2 (33)	1 (33)		22 (65)	12 (75)		14 (67)	16 (59)	
Other, including preexisting comorbidity	15 (25)	11 (24)		1 (25)	0 (0)		8 (24)	2 (13)		6 (29)	9 (33)	
Missing, unknown	7 (12)	5 (11)		1 (25)	0 (0)		4 (12)	2 (13)		1 (5)	2 (7)	

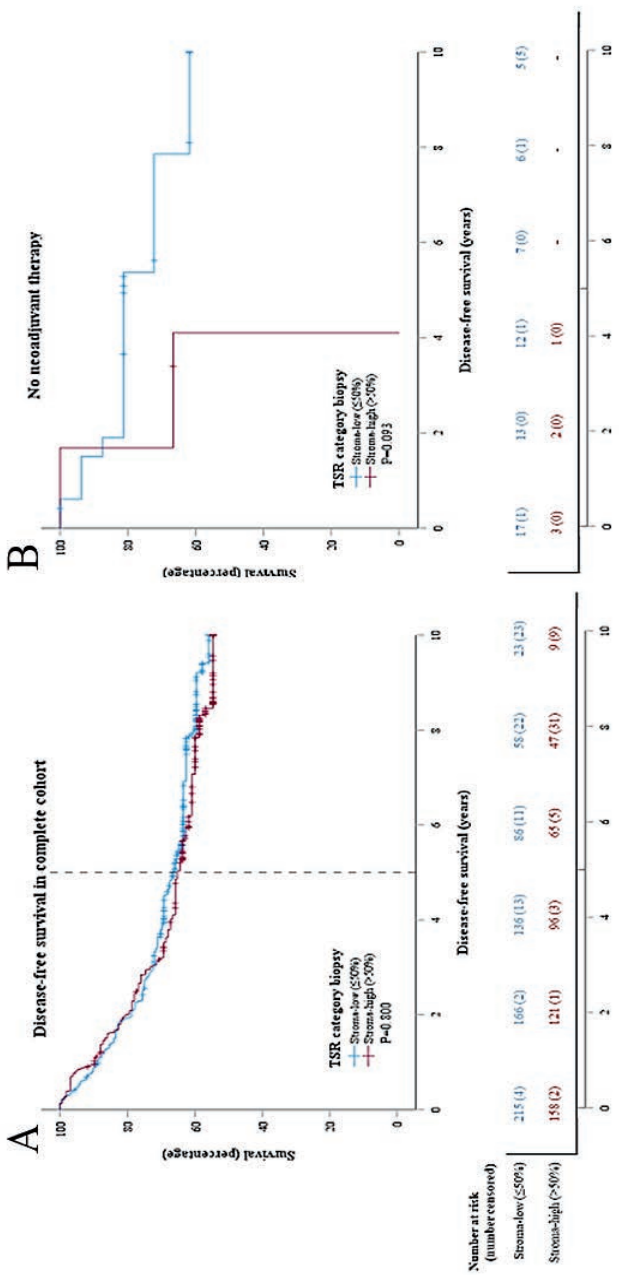
Other type of NAT (N=5) than above is excluded at category for analysis purposes. Disease-free and overall survival are censored after 10 years. All variables are given as absolute numbers with associated percentages or medians with interquartile ranges. Sum of percentages can be less or more than 100 due to rounding.

N/A, not applicable; SE, standard error; SCRT, short course radiotherapy (5x5 Gray); TNM, tumour-node metastasis; NAT, neoadjuvant treatment, including chemoradiation (25x1.8-2 Gray and capecitabine monotherapy) and RAPIDO (5x5 Gray followed by 6 cycles capecitabine and oxaliplatin).

ǂ Calculated with a reverse Kaplan-Meier analysis and log rank test.

§ Calculated with Kaplan-Meier analysis and log rank test.

Calculated using the Chi-square test.



Supplementary Figure 1. Kaplan-Meier analysis and log rank test for disease-free survival (DFS) in stroma-low rectal carcinoma patients compared to stroma-high rectal carcinoma patients. (A) In the complete cohort (5-year DFS 66% vs. 65%; P=0.800). (B) In the group without neoadjuvant therapy (5-years DFS 81% vs. none; P=0.093).

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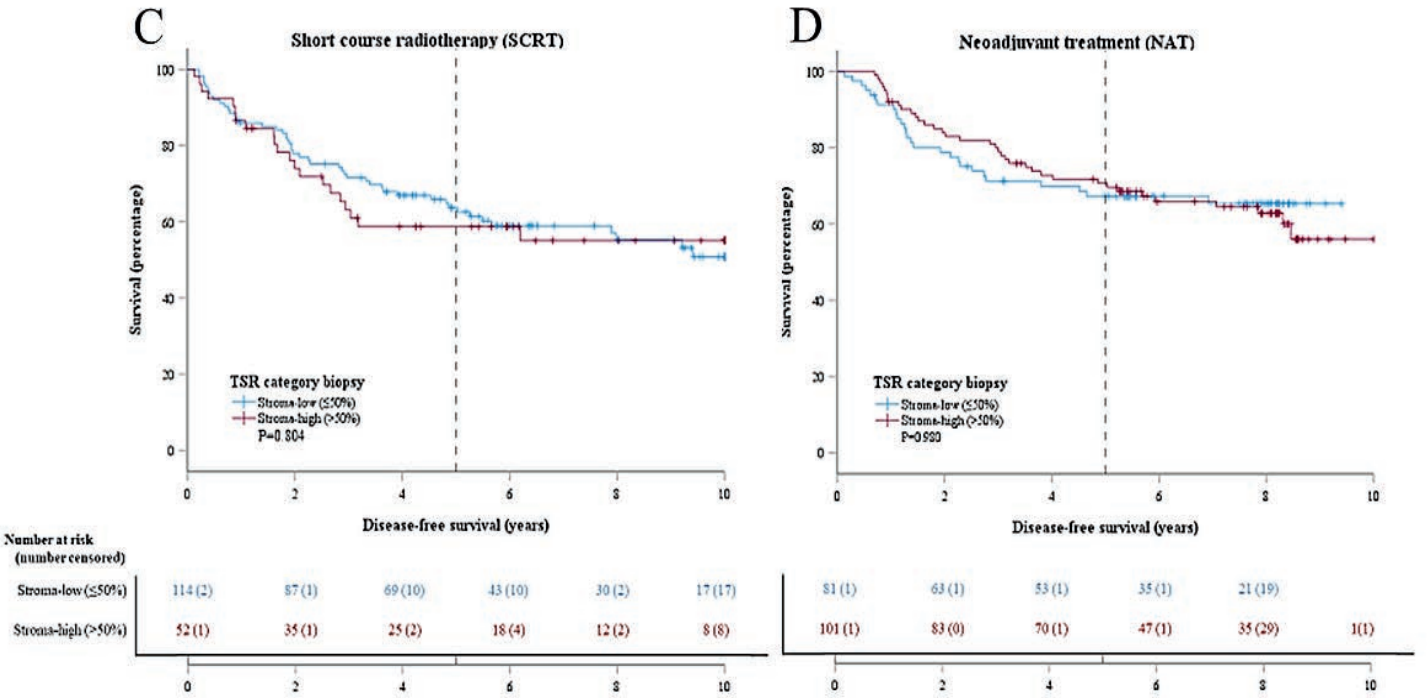
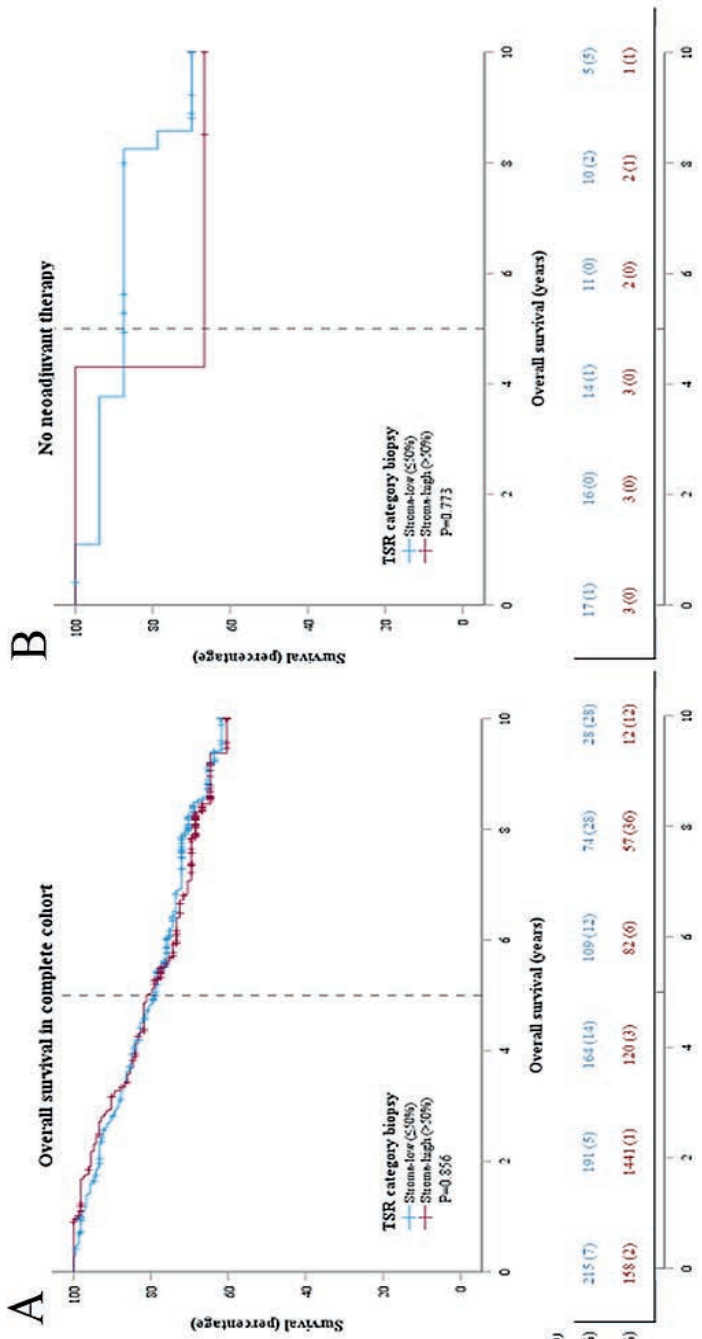
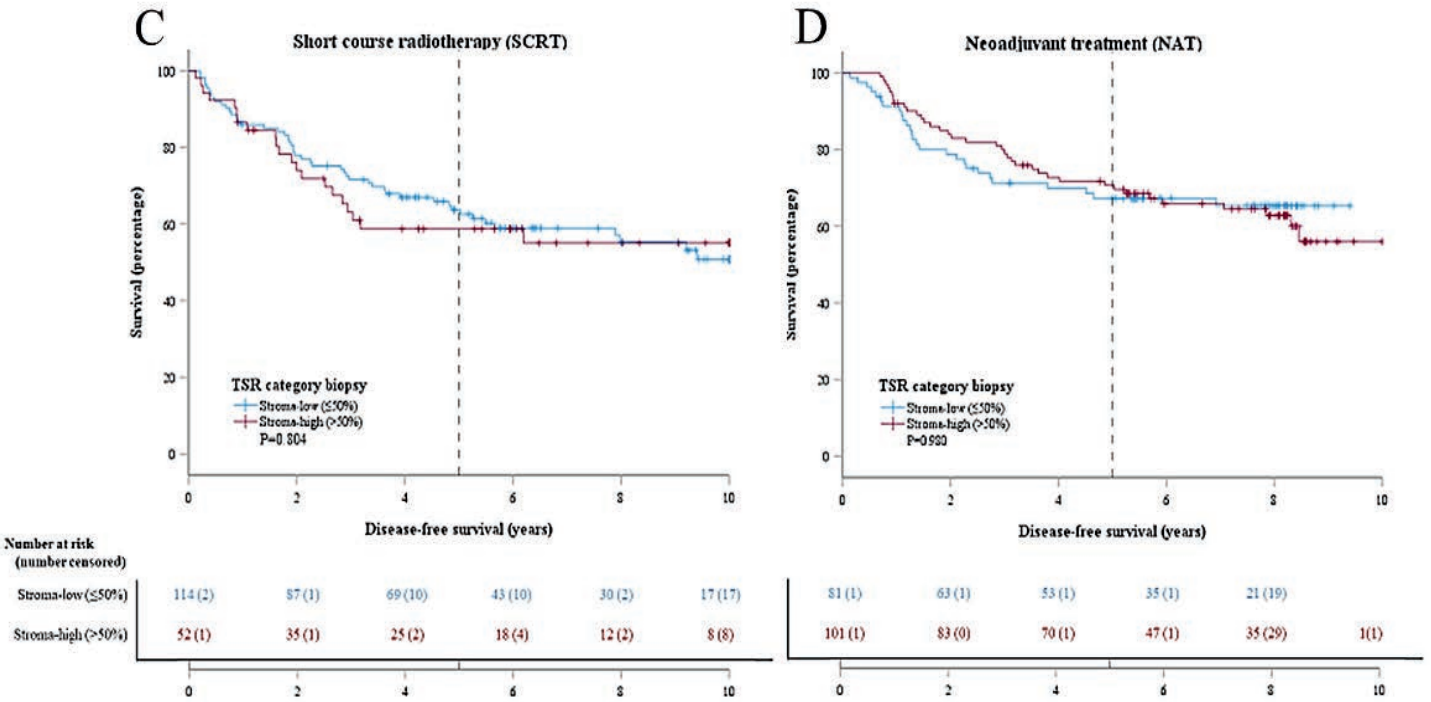


(continued) **Supplementary Figure 1.** Kaplan-Meier analysis and log rank test for disease-free survival (DFS) in stroma-low rectal carcinoma patients compared to stroma-high rectal carcinoma patients. (C) In the group with SCRT (5-year DFS 64% vs. 59%; P=0.804). (D) In the NAT group, including the chemoradiation and RAPIDO-treated patients (5-year DFS 67% vs. 71%; P=0.980). The TSR as single parameter does not reach significance in any analysis.

TSR, tumour-stroma ratio.



Supplementary Figure 2. Kaplan-Meier analysis and log rank test for overall survival (OS) in stroma-low rectal carcinoma patients compared to stroma-high rectal carcinoma patients. (A) In the complete cohort (5-year OS 79% vs. 81%; P=0.856). (B) In the group without neoadjuvant therapy (5-years OS 88% vs. 67%; P=0.773). *(continued on next page)*



(continued) **Supplementary Figure 2.** Kaplan-Meier analysis and log rank test for overall survival (OS) in stroma-low rectal carcinoma patients compared to stroma-high rectal carcinoma patients. (C) In the group with SCRT (5-year OS 77% vs. 72%; P=0.696). (D) In the NAT group, including the chemoradiation and RAPIDO-treated patients (5-year OS 80% vs. 88%; P=0.764). The TSR as single parameter does not reach significance in any analysis.

TSR, tumour-stroma ratio.